Antiviral and virucidal activities against arenaviruses of Zinc-finger active compounds

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Abstract

Fifteen antiretroviral Zn-finger active compounds with diverse chemical structures, including azoic compounds, hydrazide derivatives, disulfide-based reagents and others, were screened in vitro against Junin virus (JUNV), the etiological agent of Argentine hemorrhagic fever, by a virus yield inhibition assay in Vero cells. Cytotoxicity was evaluated simultaneously by the MTT method. From the total of compounds, three were totally inactive as antivirals, nine presented moderate anti JUNV-activity and three were truly active with EC_{50} (effective concentration 50%) values in the range 6.5-9.3 µM and selectivity indices greater than 10. The most active inhibitors, named NSC20625, 3-7 and 2-71, demonstrated a broad range of action against arenaviruses, including several attenuated and pathogenic strains of JUNV as well as the antigenically related Tacaribe virus (TACV) and Pichinde virus (PICV). The direct treatment of JUNV and TACV virions with the compounds has shown two types of behavior: the aromatic disulfide NSC20625 was a very potent virucidal agent, whereas the other two compounds exhibited moderate or negligible virus inactivating properties.

Arenaviridae is a family of enveloped viruses containing two genome single-stranded RNA segments, a large (L) and a small (S) fragment, both with ambisense coding arrangement. Only five proteins are expressed from the two genome fragments : the S RNA encodes the major structural proteins, the nucleocapsid protein (NP) and the two envelope glycoproteins (the external GP1 and the transmembrane GP2), whereas the L RNA encodes the RNA polymerase protein L and a 11-kda protein called Z or p11 with unknown function. The Z protein was the last to be described when the sequences of the L segments of lymphocytic choriomeningitis virus (LCMV) (Salvato & Shimomaye, 1989), Tacaribe virus (TACV) (Iapalucci *et al.*, 1989), and Lassa virus (Djavani *et al.*, 1997) were completed. The Z protein sequence in members of *Arenaviridae* maintains a $Cys_3HisCys_4$ RING-finger motif, a type of Zn-binding polypeptide sequence that coordinates two zinc ions (Saurin *et al.*, 1996). It has been shown that the LCMV Z protein effectively binds Zn in vitro (Salvato & Shimomaye, 1989).

Several arenaviruses are human pathogens causing severe hemorrhagic fevers. In particular, Junin virus (JUNV) is the agent of Argentine hemorrhagic fever (AHF), an endemo-epidemic disease affecting the population of the most fertile zone of Argentina (Weissenbacher *et al.*, 1987). Although several compounds were found to be selective inhibitors of the in vitro replication of JUNV (Andrei & De Clercq, 1990; Candurra *et al.*, 1996, 1999; Candurra & Damonte, 1999; Cordo *et al.*, 1999), no reliable drug therapy is presently available for the treatment of AHF. Ribavirin is the only compound that has shown partial efficacy against JUNV infections but with a high level of undesirable secondary reactions (McKee *et al.*, 1988; Enria & Maiztegui, 1994). Thus, the treatment of choice for AHF consists of the early administration of standardized doses of convalescent plasma, but this therapy is not efficient when it is initiated after 8 days of illness and a late neurological syndrome is observed in 10% of the treated patients (Enria & Maiztegui, 1994).

Zn-binding proteins with cysteine-rich Zn-finger motifs seem to represent a potential novel strategy for antiviral chemotherapy. Recent studies have identified a series of chemotypes that selectively target the retroviral Zn-finger motifs of the human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein p7 (NCp7) causing Zn ejection from the protein, loss of its native structure and inhibition of virus replication (Rice *et al.*, 1996; Tummino *et al.*, 1997; Witvrouw *et al.*, 1997). Since the Zn-binding domain in the 11 kda-Z protein of several arenaviruses is highly conserved turning this

protein an attractive target for antiviral action, in the present study we evaluated selected retroviral Zn-finger agents for their capacity to act as arenavirus inhibitors.

Fifteen compounds with known activity toward HIV-1 NCp7 Zn-fingers and representative of diverse chemical structures were provided by Dr. William Rice, National Cancer Institute-Frederick Cancer Research and Development Center, SAIC Frederick, USA. Stock solutions at a concentration of 100 mM were prepared in dimethylsulfoxide. Ribavirin (Sigma-Aldrich, USA) was assayed as a reference substance.

Antiviral activity was evaluated by a virus yield inhibition assay in Vero cell monolayers grown in 24-well microplates, and infected with JUNV, strain IV4454, in the presence of different concentrations of the compounds. After 48 h of infection, supernatant cultures were harvested and extracellular virus yields were determined by plaque formation. The effective concentration 50% (EC_{50}) and the effective concentration 90% (EC_{90}) were calculated as the concentrations required to reduce virus yield by 50% and 90%, respectively, in the compound-treated cultures compared with the untreated ones. Cytotoxicity was measured with 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) method in Vero cells grown in 96 well-plates, using conditions equivalent to those used in antiviral assays (48 h of incubation at 37°C), and results were expressed as the cytotoxic concentration 50% (CC_{50}), compound concentration required to reduce the MTT signal by 50% compared to controls.

The compounds tested were grouped in two categories according to their chemical structure, as shown in Tables 1 and 2. The first category of compounds consisted of eight diverse disulfide-based compounds. As shown in Table 1, all these compounds were moderate or highly active against JUNV, with EC_{50} values ranging from 4.6 to 100 µM. However, the thiuram disulfide 5-7 was too toxic to be considered valuable as antiviral. Among the aromatic disulfides, two compounds (NSC4493 and NSC20625) were both selective inhibitors of JUNV with selectivity indices about 10, but NSC20625 exhibited a strong efficacy because its EC_{50} was 4-fold lower than the corresponding to NSC4493. Finally, the two *cis* and *trans* isomers of 1,2-dithiane-4,5-diol, 1,1-dioxide (namely NSC624151 and NSC624152, respectively) demonstrated to be active against JUNV, although the *cis*-diol 624151 exerted a slightly superior effectivity with an EC_{50} of 17.0 µM and $CC_{50} > 400$ µM. The most active inhibitors showed similar or higher efficacy in vitro than ribavirin (Table 1).

From the total of seven azoic and hydrazide derivatives tested, three were totally inactive, two demonstrated moderate anti-JUNV activity, whereas two compounds, 3-7, 2-(2-nitroso-1-naphthylazo)benzoic acid, and 2-71, 2-(carbamoylthio)-acetic acid 2-phenyl hydrazide, were truly active toward JUNV with EC_{50} values of 6.5 and 8.9 µM, respectively (Table 2). In addition, the CC_{50} of these two compounds greatly exceeded the effective antiviral doses, thus resulting in selectivity indices of 30.0 and 58.0 for 2-71 and 3-7, respectively.

The anti-arenavirus activity of both chemical classes of compounds was comparable to their antiretroviral effect against HIV previously reported (Rice *et al*., 1996; 1997a; 1997b).

The antiviral potency of the active compounds was also evaluated by a plaque reduction assay against JUNV in Vero cells. For example, the concentrations required to reduce plaque number by 50% for NSC20625, 2-71 and the reference compound ribavirin were 5.7, 22.0 and 17.9 µM, respectively, values not much different in comparison to the EC_{50} obtained by virus yield inhibition assay (Tables 1 and 2).

Three compounds, NSC20625, 3-7 and 2-71, representatives of different chemical structures and with EC_{50} values lower than 10 µM and SI greater than 10, were further examined for their inhibitory action on arenaviruses. As shown in Table 3, these compounds effectively inhibited the replication of several strains of JUNV, including attenuated isolates such as the above mentioned IV4454 and the XJCl3 and Cl67 strains as well as the highly pathogenic XJ strain. When other species of arenaviruses were tested, the values of EC_{50} for TACV, an arenavirus closely related to JUNV as shown by antigenic cross-reactivity and in vivo protection studies (Martinez Peralta *et al.*, 1993), and for Pichinde virus (PICV) were similar to those obtained for JUNV (Table 3). In most cases, the disulfide NSC20625 was the most active compound.

As a first approach to characterize the action of these three compounds on arenaviruses, an assay of virucidal activity was performed. To this end, a virus suspension containing $1x10^{6}$ PFU of JUNV was exposed for 1.5 h at 37°C to various concentrations of compounds, and then samples were chilled and diluted to determine residual infectivity by plaque formation. The sample dilution effectively reduced the drug concentration to be incubated with the cells at least 100-fold and was below the effective antiviral concentration EC_{50}. The comparison between the antiviral action in infected cells and the inactivating effect on virus particles against two arenaviruses, JUNV and TACV, shown in Figure 1, has evidenced different types of behavior for these three agents. The

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aromatic disulfide NSC20625 was the most potent virucidal agent, reducing virus titers more than 3 log at concentrations below 5 µM. From the data of Figure 1, the inactivating concentration 50% (IC_{50}) of NSC20625 was calculated to be 0.7 and 1.3 µM against JUNV and TACV, respectively, whereas the corresponding EC_{50} values in virus yield inhibition assays were 9.3 and 3.6 µM, respectively (Table 3). The virucidal potency of NSC20625 was more clearly shown when the inactivating concentration 90% (IC_{90}) was compared to the virus yield effective concentration EC_{90}: for JUNV the IC_{90} and the EC_{90} were 1.6 and 50.0 µM, respectively, whereas for TACV the IC_{90} was 3.1 µM and the EC_{90} was 37.6 µM. By contrast, the compound 3-7 only inactivated TACV virion infectivity by 50 % at a concentration as high as 40 µM whereas its virucidal activity against JUNV was negligible. The compound 2-71 demonstrated an intermediate behavior with the same pattern of curves for antiviral and virucidal activities against both viruses (Figure 1), and the extrapolated values of IC_{50} (31.3 and 21.1 µM for JUNV and TACV, respectively) were similar to the corresponding EC_{50} shown in Table 3.

The variable ability to inactivate JUNV and TACV virions demonstrated by the compounds shown in Figure 1, independently of their virus yield inhibitory effect, prompted us to evaluate the virucidal activity of all the series of Zn-finger active drugs. Results obtained confirmed the lack of a direct correlation between antiviral effect in infected cells and the virus inactivating action of these compounds. The two dithianes NSC624151 and 624152, the aldrithiol-2 4-16 and even the azodicarbonamide (ADA) A9660, inactive in virus yield inhibition assay (Table 2), exhibited a very important virucidal activity as NSC20625, with IC_{50} and IC_{90} values 5-15 fold lower than the corresponding EC_{50} and EC_{90} doses, whereas the remaining compounds showed in the virucidal assay the same behavior as in the antiviral test (data not shown).

The major finding of the present study is the demonstration that many known antiretroviral Zn-finger active compounds with diverse chemical structures have inhibitory activity against arenaviruses. Although the majority of the most active substances were disulfide-based compounds, a more wide spectrum of related compounds must be tested to clearly establish direct structure-activity relationships.

The mode of action of Zn-finger active compounds against HIV is destruction of Zn-finger motifs in the nucleocapsid protein NCp7. At present, the mode of action against arenaviruses is unclear and further research is required to explain the different behavior of the compounds and elucidate if the compounds interact with Zn-finger domains in

arenavirus proteins or act through another mechanism. However, the ability of NSC20625 and other compounds to inactivate JUNV and TACV virions was coincidental with the virucidal activity against HIV shown by these agents, which entered the intact HIV particle and caused viral inactivation by cross-linking the NCp7 protein after Zn ejection (Rice *et al.*, 1997a; Tummino *et al.*, 1997). It is noteworthy that the disulfide compounds most active in vitro against arenaviruses had also shown the highest ability to eject Zn from the NCp7 protein of HIV as reported by Rice *et al.* (1996). Studies are in progress to test the ability of these inhibitors to eject Zn from a recombinant arenavirus Z protein and, consequently, alter its interaction with other viral proteins in virions leading to loss of infectivity.

Zn-finger proteins are essential to normal cellular function. However, it must be remarked that the noncytotoxic effect of these inhibitors was observed even for a more prolonged period of incubation, up to 6 days (data not shown). Furthermore, experimental evidence was presented that three antiviral agents that target retroviral nucleocapsid protein Zn-fingers, including the dithiane NSC624151, did not impact the functions of cellular Zn-finger proteins (Huang *et al*., 1998). Thus, the anti-arenavirus efficacy of compounds reported here without cellular toxicity in tissue culture is indicative of a true selective reactivity which enhances the future perspectives of this kind of agents to be tested in vivo against arenaviruses in mouse and guinea pig models.

Acknowledgements

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Legend to the Figure

Figure 1. Concentration-response curves for antiviral (A, B) and virucidal (C,D) activity of compounds against JUNV, strain IV4454, (A, C) and TACV (B, D). Antiviral activity was measured by virus yield inhibition assay at 48 h p.i. To determine virucidal activity virus samples were treated with the indicated compound concentrations for 1.5 h at 37°C and remaining infectivity was titrated. The data represent the average values from duplicate independent experiments.